Ergosterol in POPC Membranes: Physical Properties and Comparison with Structurally Similar Sterols

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ABSTRACT The physical properties of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/ergosterol bilayers in the liquid-crystalline phase were determined using deuterium nuclear magnetic resonance (^{2}H NMR) and vesicle extrusion. For the ^{2}H NMR experiments, the *sn*-1 chain of POPC was perdeuterated, and spectra were taken as a function of ergosterol concentration and temperature. Analysis of the liquid-crystalline spectra provides clear evidence that two types of liquid-crystalline domains, neither of which is a liquid-ordered phase, having distinct average chain conformations coexist in 80:20 and 75:25 POPC/ergosterol membranes over a wide temperature range (from –2 to at least 31°C). Adding ergosterol to a concentration of 25 mol % increases POPC-d_{31} chain ordering as measured by the NMR spectral first moment M_1 and also increases the membrane lysis tension, obtained from vesicle extrusion. Further addition of ergosterol had no effect on either chain order or lysis tension. This behavior is in marked contrast to the effect of cholesterol on POPC membranes: POPC/cholesterol membranes have a linear dependence of chain order on sterol concentration to at least 40 mol %. To investigate further we compared the dependence on sterol structure and concentration of the NMR spectra and lysis tension for several POPC/sterol membranes at 25°C. For all POPC/sterol membranes investigated in this study, we observed a universal linear relation between lysis tension and M_1. This suggests that changes in acyl chain ordering directly affect the tensile properties of the membrane.

INTRODUCTION

Sterols are important structural and functional components of the plasma membranes (PMs) of eukaryotic cells. They are amphiphilic molecules with small hydroxyl polar heads, which intercalate between the acyl chains of the phospholipids within these membranes. In particular, cholesterol, ergosterol, and phytosterols are associated with the PMs of vertebrate, fungal, and plant cells, respectively. Ergosterol is also found in the membranes of some protozoans and *Drosophila*. However, despite a burst of research activity over the last decade, particularly with respect to the ever-elusive membrane rafts, their biomembrane function is still not well understood (1–4). A particularly effective method of studying the effect of sterols on PMs is to study lipid-sterol interactions and the related phase behavior in lipid bilayer systems because lipids are the major structural building blocks of cell membranes.

The major focus of this article is the study of the physical properties of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/ergosterol bilayers in the liquid-crystalline phase. POPC is an important component of the PMs of vertebrate cells, and it is similar to the phosphoglycerolipids found in yeast cells in that it has one saturated and one monounsaturated acyl chain. POPC/ergosterol bilayers have been studied by a number of research groups. In particular, aqueous multilamellar dispersions (MLDs) of POPC/ergosterol were examined by Urbina et al. (5) using deuterium nuclear magnetic resonance (^{2}H NMR). These authors were the first to notice a complex dependence of POPC chain order on ergosterol concentration above 20 mol %. More recent studies of POPC/ergosterol have used ^{2}H NMR and the investigation of mechanical properties (6) or fluorescence polarization or anisotropy measurements using the probe diphenylhexatriene (DPH) (7,8). These studies have shown that POPC chain order, membrane bending rigidity, fluorescence polarization, and steady-state anisotropy all increase up to ~20–35 mol % ergosterol and then saturate. The saturation of the acyl chain order parameter, as determined from the first moment of the spectral intensity, M_1, for POPC/ergosterol as a function of ergosterol concentration, is in sharp contrast to its variation for POPC/cholesterol and POPC/lanosterol MLDs in the fluid phase. In both of these binary mixtures, M_1 increases with sterol concentration without saturating (5,6). Urbina et al. also found that the POPC chain-ordering effect is strongest for cholesterol and weaker for ergosterol and lanosterol, the latter being the closest stable precursor of both cholesterol and ergosterol in their respective biosynthetic (evolutionary) pathways (5).

The conformational ordering of lipid acyl chains by ergosterol and cholesterol has moreover been intensely studied for bilayers composed of lipid molecules having two saturated acyl chains, for example, dimyristoylphosphatidylcholine (DMPC) (5) and dipalmitoylphosphatidylcholine (DPPC) (9,10). It was again found, using ^{2}H NMR, that the ordering increased with increasing sterol concentration, and no

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saturation was observed below 50 mol % sterol. The increase in ordering was recently confirmed by the molecular dynamics simulations of Czub and Baginski (11) at 25 mol % sterol in DMPC. These simulations also show that the bilayer thickness of DMPC/ergosterol in the liquid-ordered phase (see below) is greater than that of DMPC/cholesterol. Because the hydrophobic thickness of the membranes is directly related to the average acyl chain order parameter (12), this implies that ergosterol orders DMPC more strongly than cholesterol. Thus the simulations are in good agreement with experimental results for these systems.

It is clear, therefore, that the saturation of the chain order parameter and of other POPC/ergosterol membrane properties is in some sense anomalous, given that ergosterol and cholesterol are similar molecules. There are slight structural differences: ergosterol has a double bond at C_7-C_8 in its fused rings and, on its flexible tail, a double bond at C_{22}-C_{23} and a methyl group at C_{24}. To investigate which of these differences gives rise to the saturation, we have used ^{2}H NMR to investigate POPC bilayers containing sterols that have mixed ergosterol-cholesterol chemical features. In particular we have used the molecule 7-dehydrocholesterol (7-DHC), which is the immediate precursor of cholesterol in its biosynthetic pathway and combines the fused ring structure of ergosterol with the flexible tail of cholesterol, to help us to determine which chemical group in ergosterol is responsible for the saturation of the ordering in POPC/ergosterol.

Next we consider the phase behavior of binary lipid/sterol MLDs. Phase diagrams for both DPPC/cholesterol and DPPC/ergosterol have been determined by ^{2}H NMR combined with differential scanning calorimetry (9,10), and the notation of Ipsen et al. (13) is used in this article to characterize the related phases as follows. The gel phase of pure phospholipid bilayers is referred to as the solid-ordered (so) phase, the liquid-crystalline phase of pure lipid bilayers is referred to as the liquid-disordered (ld) phase, and the β-phase of Vist and Davis (9), which is the liquid-crystalline phase found at higher cholesterol concentrations, is referred to as the liquid-ordered (lo) phase. The terms solid (s) and liquid (l) are used to characterize the nature of the phase, whereas the words ordered (o) and disordered (d) indicate the conformational nature of the lipid acyl chains. The DPPC/cholesterol and DPPC/ergosterol phase diagrams exhibited both so+lo and ld+lo phase coexistence regions. Thewalt and Bloom (14) showed that a phase diagram of the same character could be used to describe phase coexistence in POPC/cholesterol MLDs. Because the order parameter of POPC/ergosterol MLDs saturates at 25% ergosterol, the phase diagram for this system should exhibit characteristics that are different from those of the Vist-Davis phase diagram.

Order parameters and phase behavior of lipid membranes have also been linked to their mechanical properties. Henriksen et al. (6) studied the mechanical properties of giant unilamellar vesicles of POPC/ergosterol, POPC/cholesterol, and POPC/lanosterol by micropipette aspiration at 25°C. They found that the apparent area expansion modulus, K_a, and the bending rigidity, κ, initially increase with sterol concentration for all three binary mixtures. Furthermore, both K_a and κ saturate at ~20 mol % ergosterol for POPC/ergosterol in agreement with earlier experimental data. These authors found, in addition, that the graph of K_a versus κ for all three systems lies on a single universal curve. They then plotted K_a, κ, and K_a/κ versus the first moment, M_1, of the ^{2}H NMR spectra for all three POPC/sterol mixtures and again found that the data collapsed onto a universal curve for each physical quantity. This implies that the reduction in chain cross-sectional area associated with increased chain order and hydrophobic thickness correlates with increased membrane rigidity.

The experimental methods that we have used to study POPC/sterol membranes are ^{2}H NMR spectroscopy and vesicle extrusion. The phase behavior of POPC/ergosterol membranes was studied in detail as a function of ergosterol concentration and temperature by ^{2}H NMR. Analysis of the liquid-crystalline spectra shows that two liquid-crystalline components coexist in POPC/ergosterol membranes at intermediate ergosterol concentrations. Ergosterol is less effective than cholesterol at ordering POPC in the liquid-crystalline phase. To relate the differences in ordering between POPC/ergosterol and POPC/cholesterol to sterol structure, aqueous MLDs of *sn*-1 chain perdeuterated POPC and the ''intermediate'' sterol 7-DHC were also studied by ^{2}H NMR. The POPC chain order parameter as a function of sterol concentration was compared for the various sterols at 25°C. In addition, the lysis tensions for POPC/ergosterol, POPC/cholesterol, and POPC/lanosterol were determined as a function of sterol mole fraction at 25°C using vesicle extrusion. The effect of sterols on the properties of liquid-crystalline phase POPC membranes, as well as the correlation between acyl chain order parameters and the lysis tension, are discussed.

MATERIALS AND METHODS

POPC-d_{31}, POPC, and cholesterol (98% pure) were obtained from Avanti Polar Lipids (Alabaster, AL). Ergosterol (97% pure) and 7-dehydrocholesterol (7-DHC) (97% pure) were obtained from Fluka (Buchs, Switzerland), and 7-DHC was protected from oxidation by being kept in the dark under nitrogen. Lanosterol (97% pure) and deuterium-depleted water were obtained from Sigma-Aldrich Canada (Oakville, ON). Polycarbonate membrane filters (PCTE) with nominal pore radii of 50 and 200 nm manufactured by Whatman Nucleopore (Clifton, NJ) were purchased from VWR Canlab (Mississauga, ON).

^{2}H NMR spectroscopy

POPC-d_{31}/ergosterol MLDs were prepared for ergosterol concentrations of 0, 10, 20, 25, and 35 mol %. POPC-d_{31}/7-DHC MLDs were prepared for 7-DHC concentrations of 10, 20, and 30 mol %. POPC-d_{31} and sterol were mixed in the appropriate quantities, dissolved in benzene/methanol 4:1 (v/v), and then lyophilized. Samples were hydrated using a pH 7.4 buffer prepared in deuterium-depleted water containing 50 mM HEPES, 150 mM NaCl, and

4 mM EDTA followed by freeze-thaw-vortex cycling five times between liquid nitrogen temperature and 50°C.

^{2}H NMR experiments were performed on a Varian NMR spectrometer (Infinityplus 300) at 46.06 MHz or a locally built spectrometer at 46.8 MHz using the quadrupolar echo technique (15). The typical spectrum resulted from 10,000−15,000 repetitions of the two-pulse sequence with 90° pulse lengths of 3.95 μs, an interpulse spacing of 40 μs, and a dwell time 2 μs. The delay between acquisitions was 300 ms, and data were collected in quadrature with Cyclops 8-cycle phase cycling. The spectra were depaked using the procedure described by Lafleur et al. (16). The POPC-d_{31}/ergosterol samples were heated from −15°C to 31°C. At each temperature, the sample was allowed to equilibrate for ~20–30 min before a measurement. The spectra of POPC-d_{31}/7-DHC samples were taken at 25°C. The first moment, M_1, was calculated using

$$M_1 = \frac{1}{A}\sum_{\omega=-x}^{x} |\omega| f(\omega), \quad (1)$$

where ω is the frequency shift from the central (Larmor) frequency, $f(\omega)$ is the spectral intensity, and $A = \sum_{\omega=-x}^{x} f(\omega)$.

The spectral subtraction method (9,14,17) was used to investigate the possibility of coexisting phases within the POPC/ergosterol liquid-crystalline membranes. Within a two-phase coexistence region, each observed spectrum is a linear combination of the characteristic spectrum for each individual phase at the phase boundaries. The fraction of each phase is determined by the sample composition, and thus, the phase boundaries can be obtained using the lever rule. This calculation is valid as long as the following assumptions hold: the exchange of labeled lipid between two kinds of domains or phases must be slow enough on the NMR timescale that it can be neglected, and the domains must be large enough for the signal from lipids on the domain boundaries to be negligible.

Lysis tension determination by vesicle extrusion

The lysis tension γ_{lysis} is the tension required to rupture a lipid bilayer. We determined the lysis tension of vesicles composed of POPC and POPC/sterol mixtures using extrusion methods described previously (18). In summary, the lysis tension can be related to the minimum pressure P_{min} at which a low-concentration lipid dispersion can be pushed, or extruded, through small pores. If the pressure is not sufficient to cause lysis, large vesicles block the pores and prevent any flow through them. The relation between γ_{lysis} and P_{min} can be derived using Laplace's law for the pressure difference across a curved interface assuming a uniform tension throughout the lipid membrane (19):

$$P_{min} = 2\gamma_{lysis}\left(\frac{1}{R_p} - \frac{1}{R_v}\right). \quad (2)$$

Here R_p and R_v are the mean radii of the pores and the vesicles before extrusion, respectively. The lysis tension of the lipid membrane can be determined if R_p, R_v, and P_{min} are known.

We used an extruder (Lipex Biomembranes, Vancouver, BC) to perform these experiments. Pressure was applied using compressed nitrogen gas, and all extrusions were performed at 25°C. The pores are provided by PCTE membranes with a nominal pore size of 50 nm held within the extruder. The radius of the pores was characterized by scanning electron microscopy using a FEI Dual Beam Strata 235 Field Emission Scanning Electron Microscope. The images were analyzed, the data were displayed in a histogram, and a Gaussian distribution was fit to the data to determine the mean radius and standard deviation (SD) of the distribution of pore sizes. The mean radius and SD of the pore size distribution for the PCTE membranes used in these experiments were found to be 33.5 and 8.8 nm, respectively.

To prepare samples for extrusion, pure POPC, POPC/cholesterol, POPC/lanosterol, and POPC/ergosterol mixtures with sterol concentrations of 10, 20, and 30 mol % were hydrated in purified water at a concentration of 0.1 mg of lipid per 1 ml of water, resulting in a polydisperse suspension of multilamellar vesicles. To reduce multilamellarity and prepare these suspensions for extrusion, the samples were taken five times through a freeze-thaw-vortex process followed by a preextrusion process. The freeze-thaw process consists of freezing by immersion of the sample in liquid nitrogen, thawing by immersion of the sample in 50°C water and thorough mixing in a vortex. Preextrusion consists of passing the dispersion through the extruder containing two large-pore size PCTEs of average pore radius 200 nm.

The minimum pressure was determined from measurements of the flow rate of preextruded vesicle suspensions through small-pore PCTEs at different applied pressures. When the preextruded vesicles are pushed through small pores with a pressure larger than the minimum pressure, the flow rate of the suspension is proportional to the pressure applied, decreasing to zero as the pressure is decreased toward P_{min}. The minimum pressure was calculated from a linear fit to the plot of the flow rate as a function of extrusion pressure for each sample.

The mean radii of the preextruded vesicles were characterized by dynamic light scattering using an ALV DLS/SLS-5000 spectrometer/goniometer (ALV_Laser GmbH, Langen, Germany) and a HeNe Laser (λ = 633 nm). The light scattered from the sample was detected by a photomultiplier tube at a scattering angle of 90° from the transmitted beam. Moments-based analysis (20) was used to determine the vesicle radius from the intensity-intensity autocorrelation function.

RESULTS AND DISCUSSION

POPC-d_{31}/ergosterol membranes: ^{2}H NMR

^{2}H NMR spectra of POPC-d_{31}/ergosterol membranes were taken as a function of ergosterol concentration and temperature. The first moment, M_1, calculated from the spectra, measures the average spectral width. Liquid-crystalline and so phases have distinct ^{2}H NMR spectra; thus, the variation of M_1 with temperature can be used to probe phase changes in the membrane. Fig. 1 shows M_1 as a function of temperature, T, for all POPC-d_{31}/ergosterol MLDs. Pure POPC-d_{31}

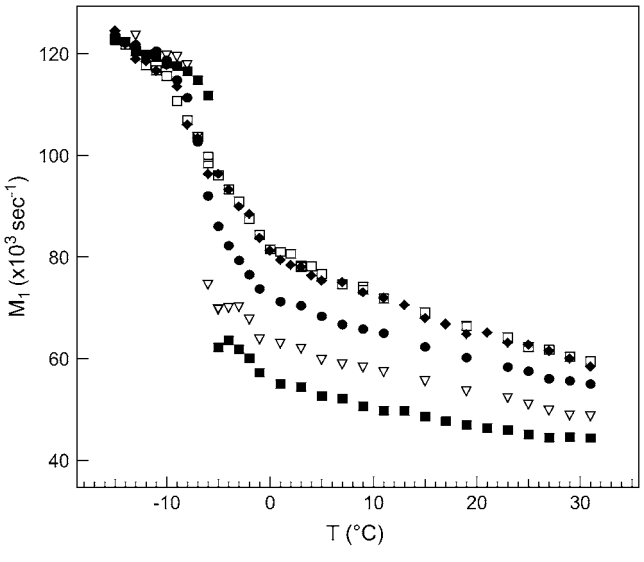

FIGURE 1 Temperature dependence of M_1 for POPC-d_{31}/ergosterol at various ergosterol concentrations: (■), 0 mol %; (∇), 10 mol %; (●), 20 mol %; (♦), 25 mol %; (□), 35 mol %.

undergoes an so-to-ld phase transition at $T_m = -5.5°C$ as the temperature is raised, manifested by a sharp reduction in M_1. The $M_1(T)$ curve plunges less dramatically at T_m as more ergosterol is added. For a given temperature above T_m, M_1 increases as the ergosterol concentration is increased from 0 to 25 mol %. Because M_1 is proportional to the average order parameter in the liquid-crystalline phase, this means that adding ergosterol increases the POPC-d_{31} chain ordering in the liquid-crystalline phase. This is also indicated by the progression of POPC-d_{31} spectra with ergosterol concentration, which is discussed next. The effect of 35 mol % ergosterol on POPC chain ordering seems to be the same as that of 25 mol %: the M_1 curve of 65:35 POPC-d_{31}/ergosterol overlaps that of the 75:25 POPC-d_{31}/ergosterol, suggesting that M_1 does not change between 25 mol % and 35 mol % ergosterol.

Fig. 2 shows the POPC-d_{31} spectra as a function of ergosterol concentration at −2°C. Pure POPC-d_{31} membranes display a characteristic liquid-crystalline spectrum with shoulders extending to ±34 kHz. When 10 mol % ergosterol is added, the spectrum becomes broader, and the shoulders extend to ±40 kHz, showing that ergosterol orders liquid-crystalline POPC membranes. The spectral width increases further up to 25 mol % ergosterol but then remains unchanged when the ergosterol concentration is increased to 35 mol %. Therefore, POPC chain ordering displays a progressive increase as more ergosterol is added until the ergosterol concentration reaches 25 mol %.

Fig. 3 shows the POPC-d_{31} spectra as a function of ergosterol concentration at 25°C. For a given ergosterol concentration, the spectral width is narrower than that at −2°C (see Fig. 2) as a result of a decrease in chain conformational order when the temperature is raised. The progression of spectral width with ergosterol concentration is similar to that in Fig. 2. The shoulders of the liquid-crystalline spectra extend to ±28 kHz for 0 mol % ergosterol and to ±39 kHz for 25 mol % and 35 mol % ergosterol. This shows that ergosterol increases the average chain ordering of POPC-d_{31} membranes, but this increase in chain ordering proceeds only up to 25 mol % ergosterol. The center of each spectrum exhibits an isotropic peak (having ~0.6% of the total intensity for pure POPC-d_{31}). This peak's intensity increases linearly with temperature; thus, we attribute it to a small amount of vesicle budding (21).

We further analyze the liquid-crystalline phase spectra of POPC-d_{31} membranes containing 20 mol % and 25 mol % ergosterol in Fig. 2, using spectral subtraction (9,14,17). A broad component and a narrow component are obtained, as presented in Fig. 4, *A* and *B*, respectively. We denote the spectra of the 20 mol % and 25 mol % ergosterol MLDs by S_{20} and S_{25}, respectively. The broad and narrow components were obtained using $S_{25} - 0.56 \times S_{20}$ and $S_{20} - 0.25 \times S_{25}$. The shoulders of the broad component, shown in Fig. 4 *A*, end at ~±55 kHz, and those of the narrow component,

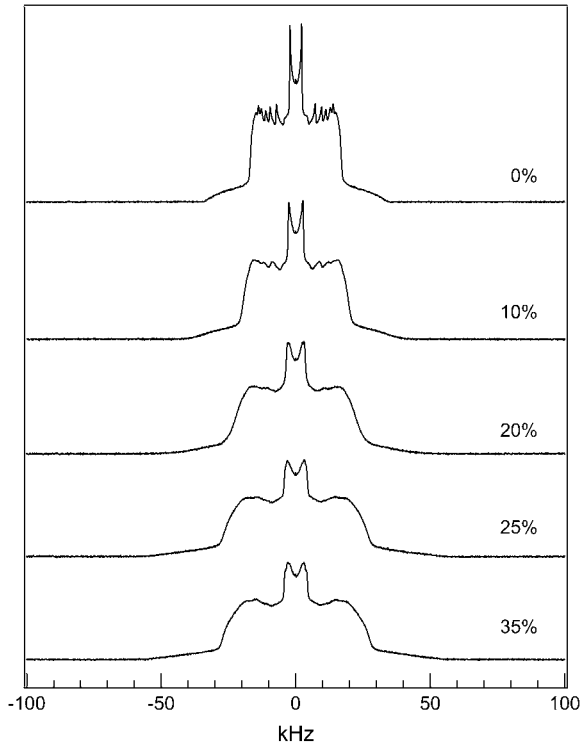

FIGURE 2 ^{2}H NMR spectra of POPC-d_{31}/ergosterol as a function of ergosterol concentration at $T = -2°C$.

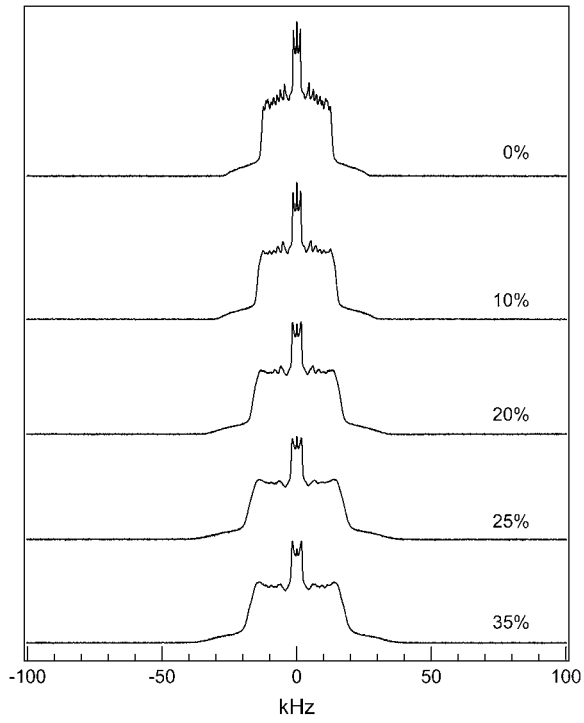

FIGURE 3 ^{2}H NMR spectra of POPC-d_{31}/ergosterol as a function of ergosterol concentration at $T = 25°C$.

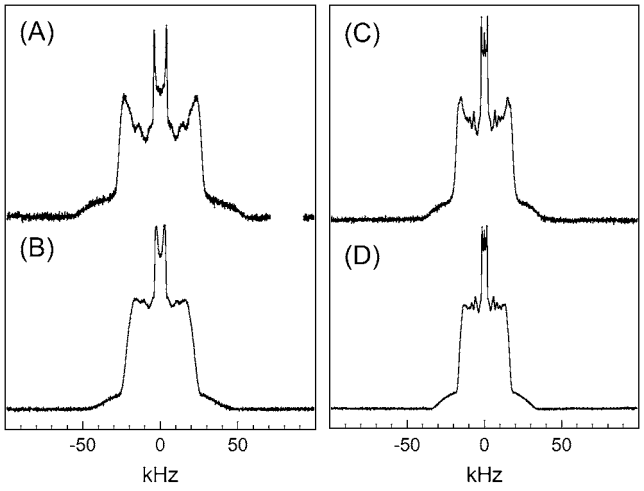

FIGURE 4 Subtracted spectra obtained from the spectral subtraction of 80:20 and 75:25 POPC-d_{31}/ergosterol membranes for (*A* and *B*) $T = -2°C$; (*C* and *D*) $T = 25°C$.

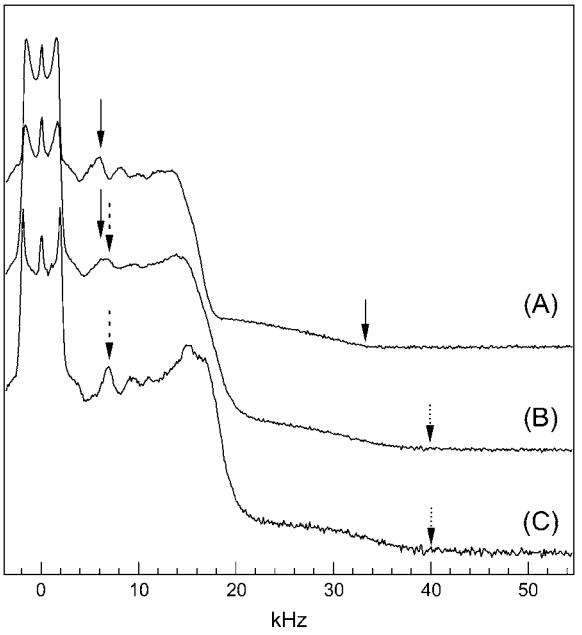

FIGURE 5 Magnified view of the spectra of the (*A*) narrow and (*C*) broad components obtained from the spectral subtraction of 80:20 and 75:25 POPC-d_{31}/ergosterol membranes at 25°C together with (*B*) the spectrum of 75:25 POPC-d_{31}/ergosterol at 25°C.

shown in Fig. 4 *B*, end at $\sim\pm45$ kHz. The average spectral width, M_1, proportional to the average chain ordering, of the broad component ($M_1 = 103{,}500\ s^{-1}$) is greater than that of the narrow component ($M_1 = 72{,}600\ s^{-1}$). Therefore, the broad component corresponds to more ordered POPC-d_{31} chains. Fig. 4, *A* and *B*, demonstrates that the 20 mol % (or 25 mol %) spectrum is a superposition of two liquid-crystalline spectra having distinct chain orderings. This suggests two types of liquid-crystalline domain coexisting in these membranes at −2°C.

The same analysis is performed on the 20 mol % and 25 mol % ergosterol spectra in Fig. 3. A broad component (with shoulders extending to $\sim\pm40$ kHz) having $M_1 = 67{,}700\ s^{-1}$ and a narrow component (with shoulders extending to $\sim\pm34$ kHz) having $M_1 = 56{,}500\ s^{-1}$ are obtained as shown in Fig. 4, *C* and *D*, respectively, indicating that the measured 20 mol % (or 25 mol %) spectrum at 25°C is also composed of two liquid-crystalline spectra.

The broad and narrow components in Fig. 4, *C* and *D*, respectively, as well as the spectrum of 75:25 POPC-d_{31}/ergosterol are plotted in Fig. 5 for better comparison. The left arrow at ~6–7 kHz of the spectrum of 75:25 POPC-d_{31}/ergosterol (Fig. 5 *B*) is indicative of the presence of the narrow component, where a peak appears in almost the same position (indicated by an *arrow* in Fig. 5 *A*). The right arrow at ~6–7 kHz of the spectrum of 75:25 POPC-d_{31}/ergosterol (Fig. 5 *B*) points to a peak characteristic of the broad component (indicated by a *dashed arrow* in Fig. 5 *C*). The shoulders of 75:25 POPC-d_{31}/ergosterol (Fig. 5 *B*) and the broad component (Fig. 5 *C*) both extend to ~40 kHz, indicated by dotted arrows, larger than that of the narrow component (Fig. 5 *A*, ~34 kHz). Therefore, 75:25 POPC-d_{31}/ergosterol is a superposition of broad and narrow components. We wish to stress that, as stated in the Methods, the proviso for determining phase coexistence, as opposed to domain coexistence, using spectral subtraction, is that the exchange of labeled lipid between phases via lateral diffusion should be sufficiently slow.

The analysis used in Fig. 4 was applied to spectra of POPC-d_{31} containing 20 mol % and 25 mol % ergosterol obtained over the temperature range –2 to 31°C. It was found that each measured spectrum is a superposition of two liquid-crystalline spectra having distinct chain orderings. This suggests that two types of liquid-crystalline domains having different ergosterol concentrations coexist in both 75:25 and 80:20 POPC-d_{31}/ergosterol membranes over a wide temperature range from –2°C to at least 31°C. Our results compare qualitatively with those of a recent fluorescence study on POPC/ergosterol membranes probed with DPH (8) in which changes in slope of the steady-state emission anisotropy as a function of ergosterol concentration were interpreted as ld or lo phase boundaries. These two liquid-crystalline phases were found to coexist from 15 to 50°C between ~10 mol % and ~40 mol % ergosterol. However, we do not assert that ld+lo phase coexistence occurs in POPC-d_{31}/ergosterol membranes because 1), the width of the more ordered component is far less than the lo spectral width in DPPC-d_{31}/ergosterol (10), and 2), spectra characteristic of the so phase are observed at 35 mol % ergosterol at temperatures close to the T_m of pure POPC-d_{31} (Fig. 1).

Attempts at spectral subtraction using the spectra of 90:10 POPC-d_{31}/ergosterol were unsuccessful. As soon as one tries to eliminate the broad component from the spectra of 90:10 POPC-d_{31}/ergosterol shown in Fig. 2 or Fig. 3, a dip at

~20 kHz appears in the spectra. We interpret this to mean that 90:10 POPC-d_{31}/ergosterol membranes are near a phase boundary, in agreement with the findings of Silva et al. (8)

It is also interesting to compare our POPC/ergosterol results with studies of the liquid-crystalline phase behavior of POPC/cholesterol membranes. For example, de Almeida et al. (22) observed two-phase coexistence for the POPC/cholesterol system above T_m using absorption and fluorescence techniques. These two liquid-crystalline phases, which were interpreted by the authors as lo and ld, coexist over a wide temperature range (from 15 to ~40°C). At 25°C, the phases coexist at cholesterol concentrations between ~13 mol % and ~45 mol %. Similarly, Mateo et al. (23) found that two liquid-crystalline phases are formed in the POPC/cholesterol system by observing the emission from the fluorescent bilayer probe *trans*-parinaric acid. These phases coexist from 10 to ~40°C and, at 25°C, were found for cholesterol concentrations between ~7 mol % and ~37 mol %. On the other hand, a fluorescence microscopy study of Texas Red dipalmitoylphosphatidylethanolamine probing POPC/cholesterol membranes containing 20 mol % or 30 mol % cholesterol showed that only one liquid phase is present between 10 and 60°C (24). Filippov et al. (25) measured the lipid lateral diffusion coefficient for POPC/cholesterol membranes using pulsed field gradient NMR and showed that for cholesterol concentrations between 0 and 48.8 mol %, the membranes were in the ld phase from 25 to 60°C. However, they did not rule out the possibility that two liquid-crystalline phases might coexist at lower temperatures. Thus, the presence or absence of phase coexistence in POPC/cholesterol liquid-crystalline membranes at temperatures well above T_m is still controversial. Our observation of two types of liquid-crystalline domains coexisting in POPC/ergosterol membranes containing 20 or 25 mol % ergosterol over the temperature range –2 to 31°C is broadly analogous to the findings of de Almeida et al. and Mateo et al. (22,23).

The subtracted spectra in Fig. 4 display line broadening of individual peaks. This can be examined closely in Fig. 6, *A* and *B*, which show the depaked spectra of Fig. 4, *C* and *D*, respectively. The line broadening of individual peaks is prominent, compared with POPC-d_{31} containing 10% ergosterol, which shows a well-resolved peak in Fig. 6 *C*. The line broadening of individual peaks implies that fast lipid exchange occurs between two different environments. Therefore, the contribution of the lipids at domain boundaries cannot be neglected. Because of this, the liquid-crystalline phase boundaries in the POPC/ergosterol phase diagram, if they exist, cannot be calculated (9,14).

The analysis used in Fig. 4 was previously applied to the spectra obtained from membranes in the ld+lo phase coexistence region of the DPPC-d_{31}/ergosterol phase diagram (10). However, these spectra could not be decomposed into two components. Hsueh et al. (10) suggested that fast lipid exchange between ld and lo domains occurs in these membranes, and thus, the domain boundary lipids are important in this system. Given the fact that the POPC/ergosterol spectrum can be separated into two components, the contribution of the domain boundary lipids in POPC/ergosterol is less significant than that in DPPC/ergosterol. This may imply a reduced rate of lipid exchange in POPC/ergosterol or larger domains in POPC/ergosterol or both as compared to DPPC/ergosterol.

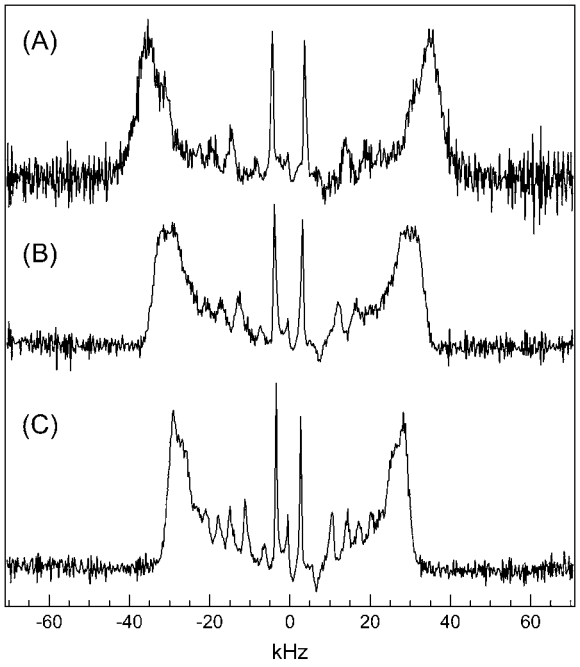

FIGURE 6 (*A*) Depaked spectra for the subtracted spectrum in Fig. 4 *C*; (*B*) the subtracted spectrum in Fig. 4 *D*; and (*C*) the spectrum of 90:10 POPC-d_{31}/ergosterol at 25°C.

POPC-d_{31}/sterol membranes: comparison of acyl chain ordering

^{2}H NMR spectra of POPC-d_{31}/7-DHC membranes were taken as a function of 7-DHC concentration at 25°C. In Fig. 7 we compare the M_1 values of POPC-d_{31} membranes containing ergosterol or 7-DHC with those of POPC-d_{31} membranes containing cholesterol (26) or lanosterol (6). For POPC-d_{31}/7-DHC, POPC-d_{31}/cholesterol, and POPC-d_{31}/lanosterol, M_1 increases with sterol concentration from 0 to 30 mol %. Because M_1 is proportional to the average order parameter of the acyl chain, this shows that these three sterols induce chain ordering over the whole concentration range investigated. The M_1 curve of POPC-d_{31}/ergosterol behaves differently from those of the other three sterols: M_1, and thus the chain ordering of POPC-d_{31} membranes, increases with ergosterol concentration up to 25 mol % but does not increase further when the ergosterol concentration is raised to 35 mol %.

The saturation in POPC membrane acyl chain order by ergosterol has previously been observed with other techniques.

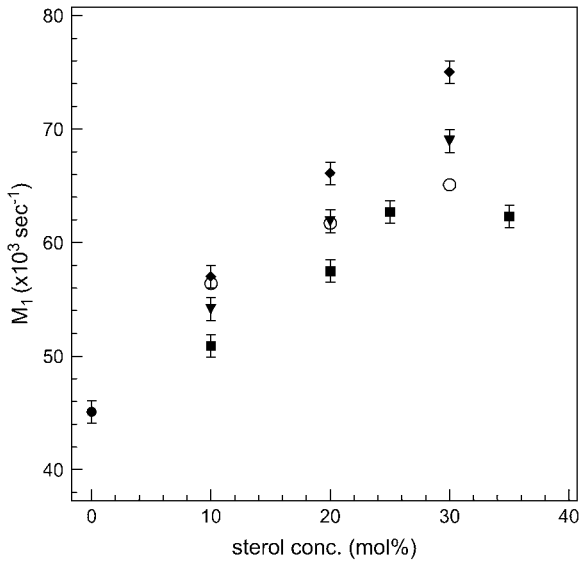

FIGURE 7 M_1 as a function of sterol concentration for (●) pure POPC-d_{31}; (■) POPC-d_{31}/ergosterol; (♦) POPC-d_{31}/cholesterol (26); (▼) POPC-d_{31}/7-DHC; and (○) POPC-d_{31}/lanosterol (6) at 25°C.

Arora et al. (7) found that at 23°C the fluorescence polarization of DPH in POPC/ergosterol membranes increased only up to 20 mol % ergosterol. ^{13}C NMR spectra revealed the presence of a second form of ergosterol for concentrations higher than 30 mol % in POPC/ergosterol bilayers (5). In addition, in a recent study of DPH fluorescence, Silva et al. (8) reported that ergosterol increased POPC membrane order only up to a concentration of 40 mol %. The precise point where saturation is observed is likely related to the source of the ergosterol: when using the same source as Arora et al. (7), we also observed saturation at 20 mol % ergosterol (data not shown). A possible explanation for these results is that ergosterol is not as soluble as, for example, cholesterol in POPC. Cholesterol is known to have a solubility limit in phospholipid membranes that depends on the level of unsaturation of the lipid acyl chains, and there have been several studies on lipid/cholesterol mixtures having cholesterol in excess of this solubility limit (27–31). The excess cholesterol is excluded from the bilayers and forms monohydrate crystals. Our results show that POPC-d_{31}/ergosterol membranes appear to have an ergosterol solubility limit at a concentration of ~25 mol %. It is therefore plausible that ergosterol in excess of 25 mol % forms ergosterol monohydrate crystals.

Fig. 7 also shows that there are differences in the magnitude of the M_1 values for different POPC/sterol mixtures. For a given sterol concentration, POPC-d_{31}/cholesterol membranes display the largest chain ordering, suggesting that cholesterol is the most effective of these four sterols in ordering POPC-d_{31} acyl chains. In contrast, POPC-d_{31}/ergosterol membranes display the smallest chain ordering, implying that ergosterol is the least effective in ordering POPC-d_{31} acyl chains. At 30 mol % sterol, M_1 decreases in the order cholesterol > 7-DHC > lanosterol > ergosterol. This observation agrees with that of Urbina et al. (5) regarding 70:30 POPC-d_{31}/sterol membranes at 25°C: they also found that cholesterol produced the largest acyl chain ordering effects, whereas 7-DHC, lanosterol, and ergosterol were progressively less effective.

These results indicate that sterol molecular structure has an impact on lipid-sterol interactions as measured through acyl-chain ordering, consistent with previous theoretical and experimental findings (32). Ergosterol, 7-DHC, and cholesterol share a common structure with small modifications; Table 1 lists the differences in the sterol structures with respect to cholesterol. 7-DHC has the ring structure of ergosterol and the methylated flexible tail of cholesterol and is less effective than cholesterol at ordering POPC chains (M_1 decreases by 6%). Ergosterol, which differs from 7-DHC only by one extra double bond in its flexible tail and a CH_3, has a smaller POPC chain-ordering effect than 7-DHC (M_1 decreases by a further 6%). Thus, a slight structural difference in either the fused ring or flexible tail of the sterol affects POPC chain ordering significantly, in agreement with the results of Urbina et al. (5). The more rigid ring and bulkier tail of ergosterol make it far less effective than cholesterol at ordering POPC acyl chains.

POPC/sterol membranes: lysis tension measurements and comparison to acyl chain ordering

The flow rate of POPC/sterol dispersions through the pores of PCTE membranes was measured as a function of applied pressure. From the minimum pressure required for extrusion, the size of the pores, and the size of the preextruded vesicles, the lysis tension of POPC/sterol vesicles containing different concentrations of cholesterol, lanosterol, or ergosterol was calculated using Eq. 2. The results are shown in Fig. 8. The figure shows that all three sterols increase the lysis tension of the POPC bilayer, with cholesterol producing the greatest effect, followed by lanosterol and ergosterol. The lysis tension of POPC/cholesterol and POPC/lanosterol bilayers continues to increase monotonically to sterol concentrations of at

TABLE 1 List of the differences in the sterol structure and the M_1 of POPC-d_{31}/sterol membranes compared to cholesterol

	Structural difference compared with cholesterol	Decrease in M_1 compared with cholesterol (66,000 s^{-1})
7-DHC	one extra double bond in the B ring between C_7 and C_8	6%
Ergosterol	one extra double bond in the B ring between C_7 and C_8 one extra double bond between C_{22} and C_{23}, and one extra CH_3 at C_{24} of the side chain	12%

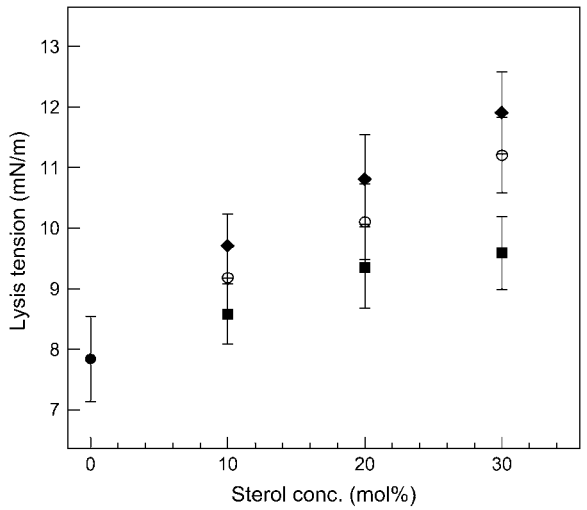

FIGURE 8 Lysis tension as a function of sterol concentration for (●) pure POPC; (■) POPC/ergosterol; (♦) POPC/cholesterol; and (○) POPC/lanosterol at 25°C. The error bars indicate the SD of the lysis tension calculated from SDs of the radii of the pores and vesicles and of the minimum pressure from the linear fitting results.

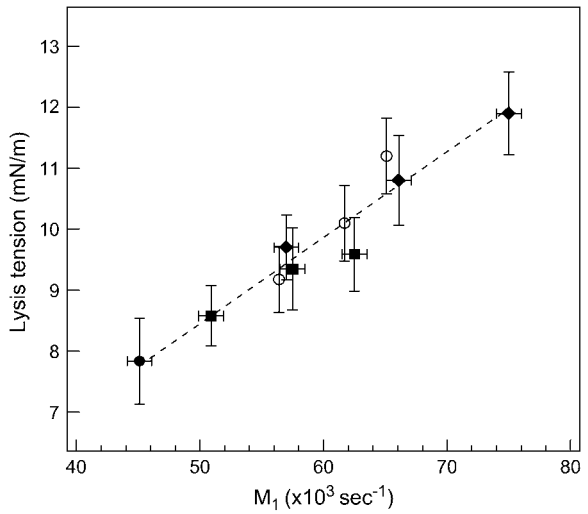

FIGURE 9 Plot of lysis tension versus M_1 for (●) pure POPC; (■) POPC/ergosterol; (♦) POPC /cholesterol; and (○) POPC/lanosterol at 25°C. The dashed line is a linear fit to the data and has a slope of 0.14 ± 0.01 μN s/m. Note that the T_m of POPC is two degrees higher than the T_m of POPC-d_{31} (−5.5°C), but because both M_1 and lysis tension are only weakly dependent on temperature near 25°C, this is a negligible correction.

least 30 mol %, whereas the lysis tension of the POPC/ergosterol bilayers appears to saturate above 20 mol %.

In Figs. 7 and 8, we observe that both the acyl chain ordering and the lysis tension of the membranes increase as the sterol concentration of the membranes is increased. Moreover, the sterols increase lysis tension and M_1 values following the same sequence: cholesterol > lanosterol > ergosterol. To elucidate this correlation, in Fig. 9 we plot these two physical quantities on the same graph and observe that the lysis tension exhibits a linear dependence on M_1, regardless of sterol. The fact that lysis tension, which represents membrane strength, is proportional to M_1, which represents acyl chain order, indicates that the properties of individual lipids affect the mechanical properties of the entire vesicle. Because M_1 is linearly related to the bilayer thickness for saturated acyl chains (12,33,34), this suggests that lysis tension is also proportional to the hydrophobic thickness of the bilayer. This is reasonable because there is competition between the applied tension that forms pores and the edge energy associated with the pore formation in the process of membrane rupture (35). The increase in the thickness of the membrane as a consequence of the presence of sterols should make pore formation more difficult because of increased edge energy.

CONCLUSIONS

The intent of this work is to identify how the physical properties of POPC/ergosterol differ from those of other POPC/sterol membranes. In particular, ergosterol has markedly different effects on POPC membranes compared with DPPC. The average acyl chain order of POPC-d_{31} is increased modestly by added ergosterol, but only up to a concentration of 25 mol %, whereas ergosterol dramatically and progressively increases the average chain order of DPPC-d_{31} up to a concentration of at least 42 mol % (10). Cholesterol, by contrast, has very similar strong chain-ordering effects on POPC and DPPC but does not order polyunsaturated PCs as effectively. We have also shown that replacing the fused ring of cholesterol by that of ergosterol to form the intermediate sterol 7-DHC somewhat reduces the sterol's capacity to order POPC-d_{31} acyl chains as compared with cholesterol. This is in agreement with the work of Urbina et al. (5). Thus, both the fused rings and the more flexible tail of the sterol are important determinants of lipid-sterol hydrophobic interactions. In particular, unfavorable interactions will limit the ability of the sterol to intercalate between the phospholipid acyl chains.

The apparent low solubility of ergosterol in the monounsaturated POPC membrane, which was also reported by Arora et al. (7), may have consequences regarding the distribution of ergosterol in yeast PMs. For example, the inability of POPC/ergosterol membranes to incorporate more than 25 mol % ergosterol may have the following consequence for raft formation in yeast PM. If we assume that yeast PM rafts are to a great extent composed of ergosterol and yeast sphingolipids, which are based on inositolphosphorylceramide, the ergosterol concentration of these rafts would be enriched to a greater extent as a result. Here we assume that the phospholipid components of the yeast PM, other than the sphingolipids, would behave like POPC in their respective lipid/ergosterol membranes because most of them have a similar hydrocarbon structure to POPC, i.e., one saturated and one unsaturated acyl chain.

Another aspect of our investigation is an NMR study of phase coexistence in POPC/ergosterol membranes. In fact, coexisting liquid-crystalline phases have been reported using fluorescent probes for both ergosterol and cholesterol in POPC membranes (8,22,23). This work presents the first clear NMR evidence that two types of liquid-crystalline domains that do not commingle extensively and have different lipid compositions can coexist in a binary lipid membrane. The liquid-crystalline spectra of 3:1 or 4:1 POPC-d_{31}/ergosterol membranes were found to consist of a superposition of two components representing membranes with distinct average chain conformational freedom at temperatures up to 31°C. In this context, it is worth pointing out that coexistence of liquid-crystalline phases has previously been observed in membranes composed of three lipids such as dioleoylphosphatidylcholine (DOPC)/DPPC/cholesterol (36), and these authors were able to calculate the phase boundaries in the ternary lipid membrane's phase diagram. A phase boundary calculation, however, was not possible in this study, leading us to conclude that exchange between domains in POPC/ergosterol is indeed influencing the subtracted spectra and thus that the coexisting domains must be smaller and/or more convoluted in shape than those in the ternary lipid membrane. It is important to emphasize that Veatch and Keller (24) do not observe liquid-liquid phase coexistence from fluorescence microscopy experiments on giant unilamellar vesicles composed of POPC/cholesterol. They also show that their data are not consistent with the fluorescence spectroscopy data of de Almeida et al. (22), which predicts two-phase regions in POPC/cholesterol. In their review article, Veatch and Keller (37) present a thermodynamic argument based on the direction of tie-lines within the two-phase coexistence region in their ternary mixtures (e.g., DOPC/DPPC/cholesterol), which does not favor the occurrence of liquid-liquid phase coexistence in DPPC/cholesterol. A theoretical study by McConnell and Radhakrishnan (38), which invokes the presence of DPPC/cholesterol complexes, agrees with this conclusion. However, POPC/DPPC/ergosterol ternary systems have not yet been investigated by the methods of Veatch and Keller. Furthermore, POPC/ergosterol membranes have different properties from POPC/cholesterol membranes (5), as illustrated in Figs. 7 and 8.

Finally, we reported a study of the lysis tension of POPC/ergosterol bilayers in comparison with those of POPC/cholesterol and POPC/lanosterol bilayers. In a related study, it was recently observed that the area expansion modulus and the bending rigidity of POPC/sterol membranes exhibited a unique functional dependence on M_1 for a variety of sterol structures and concentrations (6). The connection between the molecular descriptor M_1 and the mechanical properties of membrane vesicles has been reinforced by our observation that sterols increase both lysis tension and M_1 values in POPC following the same sequence: cholesterol > lanosterol > ergosterol. Plotting lysis tension against M_1 shows that these two properties are linearly related, with a relation that is universal to all POPC/sterol membranes observed in this study. An increase in sterol-induced POPC chain ordering affects the tensile properties of the membranes by making them more resistant to rupture. We found, however, that ergosterol is the least effective of the three sterols in increasing the resistance of POPC to rupture.

To further our understanding of lipid interactions in yeast PMs, it is important to extend these measurements to other phospholipids, such as phosphatidylethanolamine, that are prevalent in yeast. Other future investigations should include the detailed investigation of the possible formation of ergosterol monohydrate in POPC/ergosterol bilayers with higher concentrations of the sterol. Some of these studies are in progress.

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